ATENT COOPERATION TRE. .TY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2 5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day month year) 06 June 2001 (06.06.01)	
International application No. PCT US00 20519	**Applicant's or agent's file reference** 06497-013 WO1
International filing date (day month year) 27 July 2000 (27.07.00)	**Priority date** (day month year) 27 July 1999 (27.07.99)
Applicant LIAO, James, C.	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

15 February 2001 (15.02.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Charlotte ENGER
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

4

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US00/20519	International filing date *(day/month/year)* 27 July 2000 (27.07.2000)	Priority date *(day/month/year)* 27 July 1999 (27.07.1999)
International Patent Classification (IPC) or national classification and IPC IPC(7): C12N 1/21, 15/52; C12P 23/00, 5/02; C12Q 1/68 and US Cl.: 435/252.3, 252.33,67, 167, 6; 536/23.2		
Applicant FOOD INDUSTRY RESEARCH AND DEVELOPMENT INSTITUTE		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of report with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15 February 2001 (15.02.2001)	26 February 2002 (26.02.2002)
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Rebecca E. Prouty Telephone No. 703-308-0196

Form PCT/IPEA/409 (cover sheet)(July 1998)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No
PCT/US00/20519

I. Basis of the report

1. With regard to the **elements** of the international application:*

☒ the international application as originally filed.

☒ the description:
pages 1-14 as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____.

☒ the claims:
pages 15-17 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____.

☐ the drawings:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____.

☐ the sequence listing part of the description:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____.

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination(under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages NONE

☐ the claims, Nos. NONE

☐ the drawings, sheets/~~fig~~ NONE

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

*** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US00/20519

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	4, 5, 7-22, 25-35	YES
	Claims	1-3, 6, 23, 24	NO
Inventive Step (IS)	Claims	21-22	YES
	Claims	1-20, 23-35	NO
Industrial Applicability (IA)	Claims	1-35	YES
	Claims	NONE	NO

2. CITATIONS AND EXPLANATIONS (Rule 70.7)
Please See Continuation Sheet

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Claims 1-3 lack novelty under PCT Article 33(2) as being anticipated by Shin et al.

Shin et al. teach an *E. coil* comprising an *flhDC-lac* fusion which is regulated by the *OmpR* response regulator protein and acetyl phosphate levels.

Claims 1, 2, 6, and 24 lack novelty under PCT Article 33(2) as being anticipated by Haldimann et al.

Haldimann et al. teach an *E. coli* comprising a *VanH* promoter fused to a *lacZ* gene as well as a *VanS* mutation which inactivates the *VanS* histidine kinase. This fusion is regulated by the presence of *VanR* and acetyl phosphate levels.

Claim 23 lacks novelty under PCT Article 33(2) as being anticipated by Misawa et al.

Misawa et al. teach constructs for overproducing carotenoids in microorganisms such as *E.coli*.

Claims 4-9, 14-20, 24-26 and 30-34 lack an inventive step under PCT Article 33(3) as being obvious over Shin et al. or Haldimann et al. in view of McCleary et al. (1993), McCleary et al. (1994) and Liao.

Shin et al. and Haldimann et al. are discussed above.

McCleary et al. (1993) and McCleary et al. (1994) each teach that acetyl phosphate may act as a global regulatory signal in *E. coli* responsible for the activation of a wide range of response regulators of two-component systems including the *glnA* promoter in the absence of the NR_{II} protein. They further teach that acetyl-phosphate levels within a cell correlate with the amount of acetate produced.

Liao teach constructs for the expression of phosphoenol pyruvate synthase (pps) useful for increasing the amount of carbon flow into the aromatic pathway. These constructs comprise the pps gene under the control of an inducible promoter. They further show that cells lacking induction of the pps gene that acetate levels are significant but that induction of the pps gene produces undetectable levels of acetate in the cell

As inducers such as IPTG used in the constructs of Liao are expensive it would have been desirable to one of ordinary skill in the art to link the production of pps to the presence of a metabolite of the cell which signals that significant amounts of carbon are being diverted away from the aromatic biosynthetic pathway. Liao teach that acetate production occurs when there is insufficient pps expression. Therefore, it would have been obvious to one of ordinary skill in the art to replace the *tac* promoters of the constructs of

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Liao with a promoter which is induced by high acetate levels. As McCleary et al. (1993) and McCleary et al. (1994) teach that acetyl-phosphate levels within a cell correlate with the amount of acetate it would have been obvious to one of ordinary skill in the to insert the pps gene into a construct regulated by acetyl phosphate such as those of Shin et al. or Haldimann et al. or a similar construct using any of the known two-component system promoters regulated by response regulators phosphorylated by acetyl phosphate such as the *glnA* promoter.

Claims 4-13, 15-18, 24-32 and 35 lack an inventive step under PCT Article 33(3) as being obvious over Shin et al. or Haldimann et al. in view of McCleary et al. (1993), McCleary et al. (1994) and Misawa et al.

Shin et al., Haldimann et al., McCleary et al. (1993) and McCleary et al. (1994) are discussed above.

McCleary et al. (1993) and McCleary et al. (1994) each further teach that acetyl-phosphate levels within a cell correlate with the amount of acetyl-CoA produced. McCleary et al. (1993) further teach that acetyl-CoA serves as the ultimate precursor for fatty acid biosynthesis.

Misawa et al. teach constructs for overproducing carotenoids in microorganisms such as *E.coli*. These constructs comprise the carotenoid biosynthetic genes under the control of a suitable promoter.

It would have been desirable to one of ordinary skill in the art to link the production of the carotenoid biosynthetic genes to the presence of high levels of the necessary precursors for fatty acid biosynthesis. Therefore, it would have been obvious to one of ordinary skill in the art to replace the promoters of the constructs of Misawa et al. with a promoter which is induced by high acetyl-CoA levels. As McCleary et al. (1993) and McCleary et al. (1994) teach that acetyl-phosphate levels within a cell correlate with the amount of acetyl-CoA it would have been obvious to one of ordinary skill in the to insert the carotenoid biosynthetic genes into a construct regulated by acetyl phosphate such as those of Shin et al. or Haldimann or a similar construct using any of the known two-component system promoters regulated by response regulators phosphorylated by acetyl phosphate such as the *glnA* promoter. et al.

Claims 21 and 22 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest a cell expressing both a pps gene and isoprenoid biosynthetic genes together.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/20519

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 1/21, 15/52; C12P 23/00, 5/02; C12Q 1/68

US CL : 435/252.3, 252.33, 67, 167, 6; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/252.3, 252.33, 67, 167, 6; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	SHIN, S. et al. Modulation of Flagellar Expression in *Escherichia coli* by Acetyl Phosphate and the Osmoregulator OmpR. J. Bacteriology. August 1995. Vol. 177, No. 16, pages 4696-4702, see entire document.	1-3, 6, 24 ---------- 8-18, 25-32, 35
X --- Y	HALDIMANN, A. et al. Transcriptional Regulation of the *Enterococcus faecium* BM4147 Vancomycin Resistance Gene Cluster by the VanS-VanR Two Component Regulatory System in *Escherichia coli* K-12. J. Bacteriology. September 1997. Vol. 179 No. 18, pages 5903-5913, see entire document.	1-2, 6, 24 ---------- 8-17, 25-31, 35
Y	US 5,906,925 A (LIAO) 25 May 1999, see entire document.	8-9, 14-18, 24-26 30-32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 12 OCTOBER 2000	Date of mailing of the international search report 17 NOV 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer REBECCA PROUTY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998) ★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/20519

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,429,939 A (MISAWA et al.) 04 July 1995, see entire document.	23 ---- 8-13, 15-18, 24-32, 35
Y	MCCLEARY, W.R. et al. Acetyl Phosphate a Global Signal in *Escherichia coli*? J. Bacteriology. May 1993. Vol. 175, No. 10, pages 2793-2798, see entire document.	1-3, 6, 8-18, 24-32, 35
Y	MCCLEARY, W. R. et al., Acetyl Phosphate and the Activation of Two-Component Response Regulators. J. Biol. Chem. 16 December 1994. Vol. 269, No. 50, pages 31567-31572, see entire document.	1-3, 6, 8-18, 24-32, 35

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/20519

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

EAST, MEDLINE, SCISEARCH, LIFESCI, BIOTECHDS, EMBASE, CAS, NTIS, ESBIOBASE, BIOTECHNO
search terms: glnap?, acetyl phosphate or acetylphosphate, promoter?, induc? or regulat? or activat? or modulat?

PATENT COOPERATION TREATY

PCT 06497-013 WO1

EJC
YRT

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
TSAO, Y., Rocky
Fish & Richardson P.C.
225 Franklin Street
Boston, MA 02110
ETATS-UNIS D'AMERIQUE

RECEIVED
FEB 12 2001
FISH & RICHARDSON, P.C.
BOSTON OFFICE

Date of mailing (day month year) 01 February 2001 (01.02.01)	
Applicant's or agent's file reference 06497-013 WO1	**IMPORTANT NOTICE**
International application No. PCT/US00/20519	**International filing date** (day/month/year) 27 July 2000 (27.07.00)
Priority date (day/month/year) 27 July 1999 (27.07.99)	
Applicant FOOD INDUSTRY RESEARCH AND DEVELOPMENT INSTITUTE et al	

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   CN,EP,JP

   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   01 February 2001 (01.02.01) under No. WO 01/07567

No Docketing Required
Reviewed By Practice System
Initials:
Reviewed By Billing Secretary
Initials:

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, **a demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer J. Zahra
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

Form PCT/IB/308 (July 1996)

3796786

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

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1 February 2001 (01.02.2001)

PCT

(10) International Publication Number
WO 01/07567 A1

(51) International Patent Classification⁷: C12N 1/21, 15/52, C12P 23/00, 5/02, C12Q 1/68

(21) International Application Number: PCT/US00/20519

(22) International Filing Date: 27 July 2000 (27.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/145,801 27 July 1999 (27.07.1999) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 60/145,801 (CIP)
Filed on 27 July 1999 (27.07.1999)

(71) Applicant *(for all designated States except US)*: **FOOD INDUSTRY RESEARCH AND DEVELOPMENT INSTITUTE** [—/—]; P.O. Box 246, Hsinchu 300 (TW).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **LIAO, James, C.** [—/US]; 10573 Wellworth Avenue, Los Angeles, CA 90024 (US).

(74) Agent: **TSAO, Y., Rocky**; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110 (US).

(81) Designated States *(national)*: CN, JP, US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/07567 A1

(54) Title: ENGINEERING OF METABOLIC CONTROL

(57) Abstract: The invention features a method of producing heterologous molecules in cells under the regulatory control of a metabolite and metabolic flux. The method can enhance the synthesis of heterologous polypeptides and metabolites.

ENGINEERING OF METABOLIC CONTROL

Background of the Invention

The use of recombinant DNA technology has allowed the engineering of host cells to produce desired compounds, such as polypeptides and secondary metabolites. The large scale production of polypeptides in engineered cells allows for the production of proteins with pharmaceutical uses and enzymes with industrial uses. Secondary metabolites are products derived from nature that have long been known for their biological and medicinal importance. Because of the structural complexity inherent in such molecules, traditional chemical synthesis often requires extensive effort and the use of expensive precursors and cofactors to prepare the compound. In recent years, the expression of heterologous proteins in cells has facilitated the engineering of heterologous biosynthetic pathways in microorganisms to produce metabolites from inexpensive starting materials. In this manner, a variety of compounds have been produced, including polyketides, β-lactam antibiotics, monoterpenes, steroids, and aromatics.

Summary of the Invention

The invention is based, in part, on the discovery that production of heterologous polypeptides and metabolites can be enhanced by the regulated expression of the polypeptide (e.g., a biosynthetic enzyme) using a promoter which is regulated by the concentrations of a second metabolite, e.g. acetyl phosphate. The term "heterologous" refers to a polypeptide or metabolite which is introduced by artifice. A heterologous polypeptide or metabolite can be identical to endogenous entity that is naturally present. The term "metabolite" refers to a organic compound which is the product of one or more biochemical reactions. A metabolite may itself be a precursor for other reactions. A secondary metabolite is a metabolite derived from another.

Accordingly, in one aspect, the invention features a bacterial host cell containing a nucleic acid sequence comprising a promoter and a nucleic acid sequence encoding a heterologous polypeptide. Examples of bacterial host cells include *Escherichia coli, Bacillus subtilis, Salmonella typhimurium, Agrobacterium tumefaciens, Thermus thermophilus,* and *Rhizobium leguminosarum* cells. The nucleic acid sequence is operably linked to the promoter which is controlled by a response regulator protein. In other words, the nucleic acid

sequence is linked to the promoter sequence in a manner which allows for expression of the nucleotide sequence in vitro and in vivo. "Promoter" refers to any DNA fragment which directs transcription of genetic material. The promoter is controlled by a response regulator protein, for example, ntrC, phoB, phoP, ompR, cheY, creB, or torR, of *E. coli* or its homologs from other bacterial species. Further, the response regulator protein can be another member of the cluster orthologous group (COG) COG0745 as defined by http://www.ncbi.nlm.nih.gov/COG/ (Tatusov *et al. Nucleic Acids Res.* (2000); 28:33-36). In one implementation, the promoter is bound by *E. coli* ntrC. The term "ntrC" refers to both the *E. coli* ntrC protein (SWISSPROT : P06713, http://www.expasy.ch/) and its homologs in other bacteria as appropriate. As used herein, "bound" refers to a physical association with a equilibrium binding constant (K_D) of less than 100 nM, preferably less than 1 nM. An example of the promoter is the *E. coli* $glnAp_2$ promoter, e.g. a region between positions about 93 and about 323 in the published DNA sequence, GenBank accession no. M10421(Reitzer & Magasanik (1985) *Proc Nat Acad Sci USA* 82:1979-1983). This region includes untranslated sequences from the *glnA* gene. Further, a translational fusion can be constructed between coding sequences for glnA and coding sequences for the heterologous polypeptide.

The host cell is genetically modified such that the promoter is regulated by acetyl phosphate in the absence of nitrogen starvation. For example, the host cell can genetically modified by deletion or mutation of a gene encoding a histidine protein kinase, e.g., a member of COG0642 as defined by (http://www.ncbi.nlm.nih.gov/COG/; Tatusov *et al. supra.*), e.g., glnL, phoR, phoQ, creC, or envZ. In another example, the histidine protein kinase has specificity for the response regulator protein which controls the promoter. The histidine protein kinase can be encoded by *glnL*, e.g., *E. coli glnL* (SWISSPROT P06712; http://www.expasy.ch/).

Whereas the host cell is genetically modified such that the promoter is regulated by acetyl phosphate in the absence of nitrogen starvation, for heterologous polypeptide or metabolite expression, the host cell can be propagated in any desired condition, e.g., in nitrogen starvation conditions, nitrogen poor conditions, or nitrogen rich conditions.

The heterologous polypeptide encoded by the nucleic acid sequence can be a biosynthetic enzyme required for production of a metabolite. It can be a mammalian protein, e.g., a secreted growth factor, a monoclonal antibody, or an extracellular matrix component.

In yet another example, the heterologous polypeptide can be a desired antigen for use in a vaccine, e.g., a surface protein from a viral, bacterial, fungal, or protist pathogen.

Another aspect of the invention features a kit containing a nucleic acid sequence which includes a promoter controlled by a response regulator protein. The kit further optionally contains a bacterial host cell which is genetically modified such that the promoter is regulated by acetyl phosphate in the absence of nitrogen starvation. The kit can also provide instructions for their use. The nucleic acid sequence can contain a restriction enzyme polylinker located 3' of the promoter such that a sequence inserted into the polylinker is operably linked to the promoter which is controlled by a response regulator protein. In one implementation of the kit, the promoter is the *E. coli glnAp2* promoter and the bacterial host cell is an *E. coli* cell containing a mutation or deletion of the *glnL* gene.

Another aspect of the invention features a host cell containing a first expression cassette. The first expression cassette includes a promoter, such as any of those described above, and a nucleic acid sequence encoding an enzyme required for biosynthesis of a heterologous metabolite. As used herein, "enzyme" refers to a polypeptide having ability to catalyze a chemical reaction or multiple reactions. The nucleic acid sequence is operably linked to the promoter which is regulated by acetyl phosphate in the absence of nitrogen starvation. The host cell also contains additional nucleic acid sequences for expressing other enzymes required for biosynthesis of the metabolite. Such additional sequences may be endogenous sequences expressing endogenous enzymes, or introduced sequences expressing heterologous enzymes.

In one example, the heterologous metabolite is an isoprenoid, a polyhydroxyalkanoate, a polyketide, a β-lactam antibiotic, an aromatic, or a precursor, e.g., an upstream metabolite, or a derivative, e.g., a downstream metabolite, thereof. For instance, the isoprenoid can be a carotenoid, a sterol, a taxol, a diterpene, a gibberellin, and a quinone. Specific examples of isoprenoids include isopentyl diphosphate, dimethylallyl diphosphate, geranyl diphosphate, farnesyl diphosphate, geranylgeranyl diphosphate, and phytoene. Specific examples of carotenoids include β-carotene, ζ-carotene, astaxanthin, zeaxanthin, zeaxanthin-β-glucoside, phytofluene, neurosporene, lutein, and torulene. When the desired heterologous metabolite is an isoprenoid, the heterologous enzyme can be isopentenyl diphosphate isomerase, geranylgeranyl diphosphate synthase, or 1-deoxyxylulose 5-phosphate synthase. When the desired heterologous metabolite is an polyhydroxyalkanoate,

the heterologous enzyme can be 3-ketoacyl reductase, or poly-3-hydroxyalkanoate polymerase.

The host cell can be a bacterial cell, e.g., an *E. coli* cell. The host cell is optionally genetically modified by deletion or mutation of a gene, e.g., a gene encoding a histidine protein kinase, as described above. In one specific example, the host cell further contains a second expression cassette containing a nucleic acid sequence encoding phosphoenolpyruvate synthase operably linked to a promoter regulated by acetyl phosphate concentration, e.g., *glnAp2*.

Another aspect of invention features a method of producing heterologous isoprenoids in a host cell. The method includes overexpressing phosphoenolpyruvate synthase and expressing biosynthetic enzymes required for synthesis of the heterologous isoprenoid. In one implementation, a gene in the host cell encoding a pyruvate kinase or a phosphoenolpyruvate carboxylase is genetically deleted or enfeebled. In another implementation, a gene encoding phosphoenolpyruvate carboxykinase is overexpressed in the host cell. Still another aspect of the invention features a method of producing a lycopene in a host cell. The method includes expressing the following heterologous enzymes: 1-deoxy-D-xylulose 5-phosphate synthase, a geranylgeranyl diphosphate synthase, a phytoene synthase, and a phytoene saturase. In one implementation of this method, an isopentenyl diphosphate isomerase is overexpressed, e.g., using the *glnAp2* promoter. In another implementation, a phosphoenolpyruvate synthase is overexpressed, e.g., using the *glnAp2* promoter.

Another aspect of the invention features a nucleic acid sequence containing a promoter and a sequence encoding a biosynthetic enzyme required for the production of a first metabolite. The promoter is operably linked to the sequence, and is regulated by a second metabolite whose concentration is indicative of availability of a precursor for the biosynthesis of the first metabolite. In one example, the second metabolite is a waste product produced from a precursor for the biosynthesis of the first metabolite.

In one implementation, the first metabolite is a polyhydroxyalkanoate, e.g., polyhydroxybutyrate and the nucleic acid sequence encodes a biosynthetic enzyme, e.g., a 3-ketoacyl coenzyme A (coA) reductases, or a poly-3-hydroxyoctanoyl-CoA polymerase. In another case, the first metabolite is a polyketide, a β-lactam antibiotic, or an aromatic. In a yet another case, the first metabolite is an isoprenoid, e.g., an isoprenoid mentioned herein. The nucleic acid sequence can encode a biosynthetic enzyme required for isoprenoid

production, e.g., isopentenyl diphosphate isomerase, geranylgeranyl diphosphate synthase, 1-deoxyxylulose 5-phosphate synthase, phosphoenolpyruvate synthase, farnesyl diphosphate synthase, geranylgeranyl diphosphate synthase, phytoene synthase, phytoene desaturase, or lycopene cyclase. One precursor of isoprenoids can be pyruvate. Pyruvate concentrations are related to acetate and acetyl-phosphate concentrations. Accordingly, in this instance, the second metabolite is acetyl phosphate. The promoter responding to acetyl phosphate can be controlled by a response regulator protein, e.g., a response regulator protein mentioned above. Such a promoter may only respond to acetyl phosphate in a specific host cell. In a particular example, the promoter responding to acetyl phosphate concentration is bound by *E. coli* ntrC, e.g., *E. coli glnAp*$_2$ promoter.

The promoter can be regulated by cAMP. The promoter can be a bacterial promoter which binds CAP (catabolite activator protein). In mammals, the promoter can be a promoter containing a cAMP response element (CRE), which binds to the proteins CREB, CREM, or ATF-1. In yeast cells, the promoter can be a promoter regulated by cAMP, or a promoter bound by proteins Gis1, Msn2, or Msn4. Another possible regulatory signal for the promoter can be fructose 1-phosphate, or fructose 6-phosphate. The *E. coli* FruR protein regulates such promoters.

The nucleic acid sequence can be contained on a plasmid. It can also contain a bacterial origin of replication and a selectable marker. The sequence can further contain a yeast or other eukaryotic origin of replication and appropriate selectable markers, and can be integrated into the genome.

The optimization of biosynthesis of heterologous compounds in host cells is reliant on sensing parameters of cell physiology and on utilizing these parameters to regulate the biosynthesis. One standard techniques in the art is to grow cells and for the user to exogenously add an agent, e.g., an inducer, to turn on genes required for biosynthesis of the desired product. It has been widely observed that high-level induction of a recombinant protein or pathway leads to growth retardation and reduced metabolic activity. (Kurland and Dong (1996) *Mol Microbiol* 21:1-4). The practice of exogenously supplying an inducer is empirical and does not monitor the availability of resources in the cell for biosynthesis. In contrast, natural pathways rely on feedback mechanisms to control such processes. The combination of certain promoters with specific genetically defined host cells and heterologous polypeptides in this invention unexpectedly results in a highly refined and

versatile control circuit that regulates flux to heterologous polypeptide or metabolite synthesis in response to the metabolic state of the cell. Indeed, the dynamically controlled recombinant pathway provides for enhanced production, minimized growth retardation, and reduced toxic by-product formation. The regulation of gene expression in response to physiological state will also benefit other applications, such as gene therapy.

The details of one or more embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description and from the claims.

Detailed Description

The invention provides methods of engineering metabolic control, e.g., methods of utilizing promoters in specific host cells in order to optimize protein expression for either protein production or metabolite synthesis.

A central component of the invention is an expression cassette comprising a promoter and nucleic acid sequence encoding a heterologous polypeptide whose expression is desired. The expression cassette is constructed using standard methods in the art such that the coding nucleic acid sequence is operably linked, e.g., regulated by, the promoter. The promoter is chosen such that the promoter is regulated by a parameter of cell physiology or cell metabolic state. A variety of promoters can be used. In some applications the expression cassette is contained within a plasmid, such as bacterial plasmid with a bacterial origin of replication and a selectable marker. The expression cassette can be integrated into the genome of cells using standard techniques in the art.

If the expression cassette is to be used for engineering regulated production of a heterologous polypeptide during late logarithmic growth or during stationary phase, then the promoter can be chosen accordingly. For example, a promoter can be chosen that responds to small molecule signal, e.g., a second messenger, whose levels accumulate during late logarithmic growth or during stationary phase. The second messenger can be a molecule that accumulates as a precursor, an intermediate, or a waste product of a biochemical pathway. In bacteria, the small molecule signal can be a glycolysis intermediate, e.g., fructose 1-phosphate or fructose 6-phosphate or a glycolysis waste product, e.g., acetate or acetyl

phosphate. In eukaryotic cells, cAMP concentrations are a well known signal of nutrient state.

The promoter in the expression cassette can be chosen based on the results of a large scale expression analysis experiment, e.g., a gene chip experiment. Genes which are induced by acetyl phosphate can be identified by hybridizing to a microarray labeled cDNA prepared from cells in grown in acetate and comparing the signal to a reference signal, e.g., to the signal of obtained with cDNA prepared from cells in early logarithmic growth. This experiment can be performed on both prokaryotic and eukaryotic cells, e.g., bacterial, yeast, plant and mammalian cells. For an example of such an experiment in a prokaryote, see Talaat et al. (2000) *Nat Biotechnol* 18:679-82 and Oh & Liao (2000) *Biotechnol Prog.* 16:278-86. Once a gene is identified which is expressed under the desired condition, its promoter can utilized in the expression cassette. Alternatively, the experiment can be performed by the exogenous addition of a desired molecule (e.g., a precursor in a metabolic pathway) or by manipulation of experimental conditions (e.g., growth to late logarithmic phase or growth while a biosynthetic enzyme is overproduced). Promoters can be identified based on the genes induced.

In one instance, an expression cassette is used for engineering regulated production of a metabolite in a bacterial cell. The promoter can be selected which is regulated by a second metabolite whose concentration is indicative of the availability of a precursor for the biosynthesis of the first metabolite. For example, if the first metabolite is an isoprenoid which is synthesized from the precursors, pyruvate and glyceraldhyde 3-phosphate, then the second metabolite can be acetyl phosphate. In a rich environment, cells produce an excess amount of acetyl-CoA, a product of pyruvate. The excess acetyl-CoA is used to produce ATP and acetate, which is secreted as a waste product. Acetate concentration increases with cell density. Acetate, acetyl-CoA, and acetyl-phosphate concentrations are interrelated by to the following biochemical reactions:

(1) acetyl-CoA + P_i ↔ acetyl phosphate + CoA

(2) acetyl phosphate + ADP ↔ acetate + ATP

Thus, high acetyl phosphate concentration is indicative of excess acetyl-CoA and excess pyruvate. A host cell which is genetically modified by deletion or mutation of *glnL*, for example, causes ntrC function to become acetyl phosphate dependent (Feng *et al.* (1992) *J Bacteriol* 174:6061-6070). In this fashion, a promoter regulated by ntrC, e.g., the *glnAp2*

promoter, can be used to control gene expression in response to acetyl phosphate. The *glnAp2* promoter can be obtained using standard techniques in the art. For example, primers can be designed and synthesized that anneal to the *glnAp2* promoter. The polymerase chain reaction (PCR) can be used to amplify a nucleic acid fragment containing the *glnAp2* promoter. This fragment can now be used for further constructions. Likewise, an *E. coli* strain containing deletion of histidine protein kinase gene, e.g., *glnL* can be easily prepared. See Link *et al.* (1997) *J Bacteriol.*179(20):6228-6237 for a detailed description of one possible method. The sequences encoding a desired heterologous polypeptide can be cloned downstream of the *glnAp2* promoter so that it is operably linked to the promoter. A host cell with an inactivated *glnL* gene can then be transformed with the sequences. The transformed strain can be grown, and polypeptide production monitored during the course of growth. Robust protein expression can be observed at high cell densities, as in Farmer and Liao (2000) *Nat. Biotechnol* 18:533-537, the contents of which are hereby incorporated by reference.

A mammalian cell can be used as a host cell for polypeptide or metabolite production. A promoter can be selected, e.g., a promoter that responds to cAMP. Such a promoter can contain a cAMP response element (CRE), which binds to the proteins CREB, CREM, or ATF-1. Using standard techniques in the art, a desired coding sequence can be placed under control of the promoter and transformed into the mammalian cell. In some instances, the construction can be inserted into a virus, e.g., an inactivated virus. Such implementations allow for the regulated production of a protein or a metabolite produced by a heterologous biosynthetic enzyme in a gene therapy scenario. Plant cells can also be used as host cells. Again, an appropriate promoter can be chosen, e.g., a promoter than responds to a plant hormone, metabolite, or a precursor for the production of a desired metabolite. A promoter can be identified by a microarray experiment. After fusion of a desired promoter to a desired coding sequence in an appropriate vector, the construction can be electroporated into *Agrobacterium tumefaciens* and then used to transform plant cells using standard methods in the art. In still another example, yeast cells can be manipulated to express heterologous polypeptides or metabolites under metabolic control. For example, a *Saccharomyces cerevisiae* promoter can be a promoter regulated by cAMP, e.g., a promoter bound by proteins Gis1, Msn2, or Msn4. The regulation of all yeast genes in response to a variety of metabolic conditions is increasingly well studied. For example, DeRisi *et al.* (1997) *Science*

278:680-686 describe experiments following the transcriptional profile of nearly the entire *Saccharomyces cerevisiae* gene set under various metabolic conditions. Promoters regulated by a desired metabolite can be selected based on such data. The generation of yeast plasmids and the transformation of yeast are well known in the art.

A variety of metabolic pathways can be reconstructed using the expression techniques described above. For example, a pathway to produce lycopene can be introduced in *E. coli* by constructing expression vectors for the following genes: *dxs* (coding for 1-deoxy-D-xylulose 5-phosphate synthase) from *E. coli*, *gps* (coding for geranylgeranyl diphosphate (GGPP) synthase) from *Archaeoglobus fulgidus*, and *crtBI* (coding for phytoene synthase and desaturase, respectively) from *Erwinia uredovora*. These genes can reside on a single or multiple plasmids, or can be integrated into the *E. coli* chromosome. In addition, phosphoenolpyruvate synthase can be overexpressed using any method, e.g., by fusion to the *glnAp2* promoter. Isopentyl diphosphate isomerase can be overexpressed using any method, e.g., by fusion to the *glnAp2* promoter.

In another example, a pathway to produce polyhydroxyalkanoates (PHA), e.g., polyhydroxybutyrate can be implemented in *E. coli*. PHA is a family of linear polyesters of hydroxy acids with a variety of thermoplastic properties and commercial uses. *Pseudomonas aeruginosa* genes encoding 3-ketoacyl coenzyme A reductases and poly-3-hydroxyalkanoate polymerase can be placed under regulation of a desired promoter, e.g., *glnAp2*, since acetyl-CoA levels can be indicative of precursor availability for PHA synthesis.

Without further elaboration, it is believed that the above description has adequately enabled the present invention. The following examples are, therefore to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. All publications cited herein are hereby incorporated by reference in their entirety.

Methods

Growth conditions. All *E. coli* strains were grown in shake flasks containing the designated medium at 37°C in waterbath shakers (Model G76; New Brunswick Scientific, Edison, NJ). The cultures were grown in minimal media consisting of either M9 defined salts 34 containing 0.5% (wt/vol) glucose or YE defined salts containing 1.5% (wt/vol) glucose. YE

defined salts consisted of (per liter) 14 g K_2HPO_4, 16 g KH_2PO_4, 5 g $(NH_4)_2SO_4$, 1 g $MgSO_4$, and 1 mg thiamine. Cell turbidity was monitored spectrophotometrically at 550 nm.

Metabolite measurements. Acetate, pyruvate, and other organic acids were measured using HPLC (Constametric 3500 Solvent Delivery System and Spectromonitor 3100 Variable Wavelength Detector; LDC Analytical, Riviera Beach, FL) over an organic acids column (Aminex HPX-87H, Bio-Rad Laboratories, Hercules, CA) maintained at 65°C. The mobile phase consisted of 0.01 N H2SO4, and its flow rate was kept at 0.6 ml min^{-1}. Peaks coming off the column were detected at 210 nm. Glucose was measured using Sigma kit no. 315-100. To quantify lycopene, 1 ml of bacterial culture was extracted with acetone, centrifuged, and the supernatant absorbance was measured at 474 nm. Lycopene concentrations were calculated by comparing absorbances to a standard curve.

SDS–PAGE and enzyme assays. The protocol for SDS–PAGE is as described by Laemmli (1970) *Nature* 227:680-685. Measurement of β-galactosidase activity was carried out essentially as described by Miller (1992) *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.

Results

Usage of the glnAP2 promoter in *E. coli* in a heterologous fusion to *lacZ*.

Increasing levels of acetyl phosphate can be an indicator of excess glucose flux. The current invention features host cells, nucleic acids sequences, and methods of utilizing acetyl phosphate as a signal to regulate the expression of rate-controlling enzymes in a desired metabolic pathway, both to utilize fully the excess carbon flux and to redirect the flux away from the toxic product, acetate.

In order to examine the potential of *glnAp2* as a dynamic controller of product expression, a nucleic acid sequence was constructed containing a heterologous *lacZ* gene operably linked to the *glnAp2* promoter. The *glnAp2* promoter region containing the promoter and two ntrC-binding sites can be easily obtained by standard methods known in the art. The *glnAp2* promoter was PCR-amplified from *E. coli* genomic DNA using the forward primer 5′-CAGCTGCAAAGGTCATTGCACCAAC (containing an engineered *Pvu*II site) and the reverse primer 5′-GGTACCAGTACGT-GTTCAGCGGACATAC (containing an engineered *Kpn*I site). These two primers amplified a region between positions 93 and 343 in the published DNA sequence 16 (GenBank accession No. M10421).

The *glnAp2* PCR fragment was also cloned into the *Eco*RI site of pRS551, thus generating p2GFPuv; which contains *glnAp2* in front of a promoterless *lacZ* gene. The *glnAp2-lacZ* region was transferred to λRS45 via homologous recombination (Simons *et al.*(1987) *Gene* 53:85-96), generating phage λp2GFPuv. JCL1595 and JCL1596 were constructed by integrating a *glnAp2-lacZ* fusion via infection (Silhavy *et al.* (1984) *Experiments with Gene Fusions,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY) with λp2GFPuv phage into the chromosomes of BW13711 (*lacX74)* and BW18302 (*lacX glnL2001;* Feng *et al. supra)*, respectively. This strain contains the *glnL2001* allele, which consists of an internal deletion between codons 23 and 182 of the *glnL* coding sequence and presumably results in a null mutation (Feng *et al. supra*).

The time course of the β-galactosidase (β-gal) activity was measured in wild-type and in the *glnL* mutant. The *glnAp2*-β-gal activity increases in a time-dependent fashion similar to the excreted acetate concentration from the *glnL* host (JCL1596), whereas no induction of promoter activity was found for the isogenic wild-type control (JCL1595).

Table 1. β-galactosidase activity of *glnAp2-lacZ*

	β-galactosidase activity (nmol/min-mg protein)	
	6 hours	11 hours
glnAp2-lacZ in WT (JCL1595)	<100	~100
glnAp2-lacZ in *glnL* (JCL1596)	~700	~1500
P_{lac}-*lacZ* in (VJS632)	~500	~550

Thus, in the absence of *glnL*, *glnAp2* is capable of responding to the excess carbon flux that is indicated by acetate excretion. As the cells approached the late-exponential phase, the biosynthetic requirement decreased and the cells began to exhibit an excess carbon flux, as demonstrated by the increased generation of acetate. At this point, at approximately 6 hours, unexpectedly *glnAp2*-β-gal activity began to rise to (~700 nmol/min-mg protein, see Table 1) whereas *glnAp2*-β-gal activity in the wild-type strain (JCL1595) was relatively low and remained constant throughout (~100 nmol/min-mg protein, Table 1). After more than 10 hours, *glnAp2*-β-gal activity in the absence of *glnL* was a remarkable ~1500 nmol/min-mg protein (Table 1). The induction profile of *glnAp2* is also in stark contrast to that of the *lac* promoter (P_{lac}). Chromosomal P_{lac} activity in strain VJS632 (lac^+) rapidly increased after

induction with IPTG (isopropyl-β-D-thio-galactopyranoside) and achieved a constant level of expression in the cell (~550 nmol/min-mg protein, see Table 1), which is independent of the growth phase.

Usage of the glnAP2 promoter in *E. coli* in a heterologous fusion to *pps* and *aroG*

Expression of two different metabolic enzymes, phosphoenolpyruvate synthase (*pps*) and 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase (*aroG*) were placed under the control of the *glnAp2* promoter. As controls, these same two proteins also were overexpressed from the *tac* promoter (P_{tac}), which exhibits static control, under the same genetic background and environmental conditions. Standard methods of expressing *pps* leads to growth retardation (Patnaik *et al.* (1992) *J Bacteriol* 174:7527-7532).

Plasmid pAROG was constructed by cloning a PCR fragment containing *aroG* pRW5*tkt* into the *Eco*RI-*Bam*HI sites of pJF118EH. Plasmid pPS706 has been previously described in Patnaik *et al. supra*. Both plasmid express the respective genes under the P_{tac} promoter. The PCR fragment containing the *glnAp2* promoter was cloned into the *Eco*RV-*Eco*RI sites of plasmids pAROG, and pPS706 to generate plasmids p2AROG3, and pPSG706, respectively containing the respective genes under the *glnAp2* promoter.

Host strain BW18302 (*lacX glnL2001)* was transformed with all four plasmids. The strains with the respective plasmids were grown in M9 salts-glucose media. Growth was compared after 5 hours.

Table 2. Growth of Overexpressing Strains

	OD_{550} after 5 hours growth
No plasmid	~ 0.5
P_{tac}-*aroG*	~ 0.5
glnAp2-aroG	~ 0.5
P_{tac}-*pps*	~ 0.12
glnAp2-pps	~ 0.4

As previously demonstrated, overexpression of *pps* using P_{tac}-*pps* caused marked growth retardation. However, the use of *glnAp2* unexpectedly resulted in close to normal growth (Table 2). After 15 hours, proteins were isolated from each strain and analyzed on a

10% SDS-PAGE gel. At least 500% more pps protein was expressed when the *pps* gene was controlled by the *glnAp2* promoter compared to the P_{tac} promoter. In another surprising finding, AroG protein, whose conventional overexpression is not overtly detrimental, was also at least 300% more abundant in extracts from cells utilizing *glnAp2* promoter for expression compared to the P_{tac} promoter.

Production of Lycopene in *E. coli* by *idi* Overexpression

We reconstructed a recombinant lycopene pathway in *E. coli* by expressing the genes *dxs* (coding for 1-deoxy-D-xylulose 5-phosphate synthase) from *E. coli*, *gps* (coding for geranylgeranyl diphosphate (GGPP) synthase) from *Archaeoglobus fulgidus*, and *crtBI* (coding for phytoene synthase and desaturase, respectively) from *Erwinia uredovora*. These genes were inserted into pCL1920, a low-copy-number plasmid, to form pCW9, and simultaneously overexpressed.

We used the *glnAp2* promoter to control the expression of *idi* (isopentenyl diphosphate isomerase). Constructs containing the *idi* gene were derived from a promoterless vector, pJF118, The *glnAp2* promoter was inserted to form p2IDI. As a control, the P_{tac} promoter was inserted to form pTacIDI. These plasmids were separately introduced into a *glnL* strain (BW18302) containing pCW9. The p2IDI-containing strain (*glnAp2-idi*) produced 100 mg L^{-1} lycopene after 26 h in a defined medium containing glucose. The strain containing P_{tac}-*idi* on the other hand, produced only a small amount of lycopene, (< 5 mg L^{-1}) under identical conditions. Additionally, the p2IDI strain produced almost threefold less acetate than pTacIDI, which indicates that the carbon flux to acetate was being rechanneled to lycopene.

Table 3. Carbon yield of lycopene formation in batch cultures of *E. coli*.

	Lycopene Carbon yield on glucose (mol C/mol C)
Host only (BW18302)	0.0000
+ pTacIDI (Ptac-*idi*)	0.0003
+ pTacIDI (Ptac-*idi*) / pPS184 (Ptac-*pps*)	0.0012
+ p2IDI (*glnAp2-idi*)	0.014
+ p2IDI (*glnAp2-idi*) / pPSG184 (*glnAp2-pps*)	0.022

Use of *pps* to Enhance Lycopene Yield

pps was overexpressed from *glnAp2* from another compatible plasmid, pPSG18 while the remainder of the lycopene pathway (*dxs, gps, crtBI*) was expressed using pCL1920. Coexpression of *pps* and *idi* with the lycopene pathway increased the final titer of lycopene by 50% and caused the productivity to increase by threefold, from 0.05 mg mL^{-1} h^{-1} to 0.16 mg mL^{-1} h^{-1} (Table 3) This is in contrast to the companion strain containing both pTacIDI and pPS184 (P_{tac}-*idi* + P_{tac}-*pps*), where no significant improvement in yield was observed and substantial growth inhibition occurred.

Additional Host Cells for Lycopene Production

The *pykF::cat* and *pykA::kan* alleles were introduced into a wild-type strain, in order to generate two single mutants (JCL1610 (*pykF*) and JCL1612 (*pykA*)) and one double mutant strain (JCL1613 (*pykF pykA*)) (Ponce *et al.* (1995) *J Bacteriol*177:5719-5722). The double mutant strain was able to achieve a final lycopene titer of about 14 mg lycopene/g dried cells, while the single mutant strains each obtained lycopene titers of about 2.5 mg lycopene/g dried cells. The single *pyk* mutants produced lycopene at a level similar to the wild type strain,~ 4 mg lycopene/g dried cells. Further, overexpression of Pck, phosphoenolpyruvate carboxykinase , increased the final lycopene titer by about 3-fold. Overexpression of Ppc, phosphoenolpyruvate carboxylase, reduced lycopene production by about 30%.

Other Embodiments

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and the scope of the present invention. Accordingly, other embodiments are within the scope of the following claims. For example, all homologs of the mentioned polypeptides and genes are within the scope of this invention.

What is claimed:

1. A bacterial host cell comprising a nucleic acid sequence comprising a promoter and nucleic acid sequence encoding a heterologous polypeptide; the nucleic acid sequence being operably linked to the promoter which is controlled by a response regulator protein; the host cell being genetically modified such that the promoter is regulated by acetyl phosphate in the absence of nitrogen starvation.

2. The host cell of claim 1 wherein the bacterial cell is an *E. coli* cell.

3. The host cell of claim 1 wherein the promoter is controlled by a response regulator protein selected from the list consisting of ntrC, phoB, phoP, ompR, cheY, creB, and torR.

4. The host cell of claim 3 wherein the promoter is bound by ntrC.

5. The host cell of claim 4 wherein the promoter is *glnAp2*.

6. The host cell of claim 1 wherein the host cell is genetically modified by deletion or mutation of a gene encoding a histidine protein kinase.

7. The host cell of claim 6 wherein the histidine protein kinase is encoded by glnL.

8. The host cell of claim 1 wherein the heterologous polypeptide is a biosynthetic enzyme required for production of a metabolite.

9. A host cell comprising a first expression cassette comprising a promoter and a nucleic acid sequence encoding a first enzyme required for biosynthesis of a heterologous metabolite; the nucleic acid sequence being operably linked to the promoter which is regulated by acetyl phosphate in the absence of nitrogen starvation; and nucleic acid sequences expressing other enzymes required for biosynthesis of the metabolite.

10. The host cell of claim 9 wherein the metabolite is an isoprenoid.

11. The host cell of claim 10 wherein the isoprenoid is a carotenoid.

12. The host cell of claim 10 wherein the isoprenoid is lycopene, β-carotene, astaxanthin, or one of their precursors.

13. The host cell of claim 10 wherein the first enzyme is isopentenyl diphosphate isomerase, geranylgeranyl diphosphate synthase, or 1-deoxyxylulose 5-phosphate synthase.

14. The host cell of claim 9 wherein the first enzyme is phosphoenolpyruvate synthase.

15. The host cell of claim 9 wherein the host cell is a bacterial cell.

16. The host cell of claim 15 wherein the bacterial cell is an *E. coli* cell.

17. The host cell of claim 15 wherein the cell is lacking a functional histidine protein kinase gene.

18. The host cell of claim 15 wherein the promoter is controlled by ntrC, phoB, ompR, cheY, creB, phoP, or torR.

19. The host cell of claim 18 wherein the promoter is bound by ntrC.

20. The host cell of claim 19 wherein the promoter is *glnAp2*.

21. The host cell of claim 10 wherein the host cell further contains a second expression cassette comprising a nucleic acid sequence encoding a phosphoenolpyruvate synthase operably linked to a promoter which is regulated by acetyl phosphate concentration.

22. A method of producing heterologous isoprenoids in a host cell comprising overexpressing a heterologous phosphoenolpyruvate synthase; and expressing biosynthetic enzymes required for synthesis of the heterologous isoprenoid.

23. A method of producing a lycopene in a host cell comprising expressing a heterologous 1-deoxy-D-xylulose 5-phosphate synthase, a heterologous geranylgeranyl diphosphate synthase, a heterologous phytoene synthase, and a heterologous phytoene desaturase.

24. A kit comprising a nucleic acid sequence containing a promoter controlled by a response regulator protein such that the promoter is regulated by acetyl phosphate in a defined host cell; and the defined host cell which is genetically modified by deletion or mutation of a histidine protein kinase gene.

25. A nucleic acid sequence comprising a promoter and a sequence encoding a biosynthetic enzyme required for the production of a first metabolite, the sequence being operably linked to the promoter which is regulated by a second metabolite whose concentration is indicative of availability of a precursor for the biosynthesis of the first metabolite.

26. The nucleic acid sequence of claim 25 wherein the second metabolite is a waste product produced from a precursor for the biosynthesis of the first metabolite.

27. The nucleic acid sequence of claim 25 wherein the first metabolite is an isoprenoid.

28. The nucleic acid sequence of claim 27 wherein the isoprenoid is a carotenoid.

29. The nucleic acid sequence of claim 28 wherein the isoprenoid is lycopene, β-carotene, astaxanthin, or one of their precursors.

30. The nucleic acid sequence of claim 25 wherein the second metabolite is acetyl phosphate, cAMP, fructose 1-phosphate, or fructose 6-phosphate.

31. The nucleic acid sequence of claim 30 wherein the second metabolite is acetyl phosphate.

32. The nucleic acid sequence of claim 31 wherein the promoter is controlled by ntrC, phoB, ompR, cheY, creB, phoP, or torR.

33. The nucleic acid sequence of claim 32 wherein the promoter is bound by ntrC.

34. The nucleic acid sequence of claim 33 wherein the promoter is *glnAp2*.

35. The nucleic acid sequence of claim 27 wherein the biosynthetic enzyme is isopentenyl diphosphate isomerase, geranylgeranyl diphosphate synthase, 1-deoxyxylulose 5-phosphate synthase, or phosphoenolpyruvate synthase.